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(54) **Method for preparation of hepatitis B surface antigen from human plasma**

(57) This invention relates to a method for preparing hepatitis B surface antigen, comprising treating human plasma to effect delipidization of the plasma and partial removal of plasma proteins, digesting the resulting material with pepsin at a ratio of from 0.01 to 0.5 mg of pepsin per mg of protein, filtering the digested material on a molecular filter in a phosphate buffered medium of pH 7.1 to 7.3, recovering a fraction of up to 100,000 Daltons and treating the said fraction with formaldehyde to disinfect same.

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SPECIFICATION

Method for preparation of hepatitis B surface antigen from human plasma

This invention relates to a method for preparing hepatitis B surface antigen, thereafter referred to herein as HBsAg, from human plasma. The HBsAg is a viral protein occurring in the blood of people suffering from infectious hepatitis, Type B, or present in the blood of carriers of hepatitis B virus. HBsAg on being purified from the remaining plasma proteins constitutes a vaccine for infectious hepatitis B, which on being administered to a man or an animal causes the production of HBs antibodies within the patient's body. Said antibodies protect the body from full infection caused by the infectively active particle of hepatitis B virus.

There is already known a method for preparing pure HBsAg by multiple centrifugation of suitable prepared plasma in density gradients in saccharose or caesium chloride. By this method, preparations of HBsAg can be obtained free of resting plasma proteins. The ultracentrifugation method is very time-consuming, requiring numerous expensive apparatuses to be employed. It is therefore utilized for obtaining small amounts of the antigen for research purposes only. Thus, this is a laboratory method only and is not a suitable production procedure. By this it is not possible to obtain sufficient quantities of material necessary for vaccinating even a relatively small group of persons.

Further, there are known other methods for preparation of HBsAg, consisting of filtration on gel packed columns, affinity, chromatography, and electrophoresis. None of these methods however produce completely purified antigen preparations of laboratory quality, and they are of low productivity.

The object of the present invention is to obviate and mitigate the aforementioned disadvantages.

According to the invention, there is a method for preparing pure HBsAg wherein the initial material, after lipid removal and partial removal of human plasma proteins, is digested with pepsin in a ratio of 0.01—0.50 mg/mg of protein, preferably of 0.05 mg/mg of protein. The residue is filtered off in a phosphate buffered medium of ph 7.1 to 7.3 through molecular filters, allowing the passage of molecules of up to 100,000 Daltons. The infectivity is subsequently deactivated by formaldehyde, preferably in a proportion of 1:2000 by volume, for 96 hours.

The method according to the invention, unlike other known methods, yields HBsAg completely free from plasma proteins. The said antigen when employed as a vaccine for the infectious hepatitis, Type B, does not cause any side effects in the patient vaccinated. Moreover, the method according to the invention can be utilized in bulk production of vaccine from the plasma containing hepatitis B virus protein.

The preparation of the purified hepatitis B surface antigen in the method according to the invention proceeds as follows.

In the first phase the human plasma is delipidized. 100 ml of 0.1 mole of $MnCl_2 \cdot 4\ H_2O$ is added to 1000 ml of plasma obtained from donors of HBsAg, having the titer as determined in the immunoelectroosmoprecipitation IEOP of at least 1:10.

After addition of manganese chloride the plasma is stirred in an electromagnetic agitator in an ice bath for 1 hour. The material is then centrifuged for 30 minutes at 6000 rpm. After centrifugation the pellet containing no HBsAg is discarded.

The supernatant obtained after centrifugation is brought to pH 5.6 using 0.1 M HCl and polyethylene glycol 6000 is added in the ratio of 75 g per 1000 ml of the material. The material is then stirred about 12 hours at a temperature of 4°C in an electromagnetic agitator. The material after being discharged from the agitator is centrifuged for 30 minutes at 6000 rpm. The supernatant containing no antigen is rejected, and the pellet is retained for further processing.

This pellet is dissolved in 200 ml of 0.9 NaCl, and the solution is made-up with deionized water to the original volume of the material before centrifugation. After addition of NaCl a further sediment forms in the solution. This solution is once more centrifuged for 30 minutes at 6000 rpm. After centrifugation the supernatant containing the HBs antigen is collected and the pellet rejected. Deionized water is added to the supernatant to bring the volume up to 10,000 ml. Any sediment remaining after dilution is removed by centrifuging for 30 minutes at 6000 rpm.

During the next stage of the antigen preparation process, the supernatant obtained is utilized.

The material thus delipidized is now enzymatically digested. 10,000 ml of the material is heated to 37°C, and its pH standardized to 2.5, by the addition of 1N HCl. To this pepsin Sigma is added in the ratio of 0.05 mg per 1 mg of the protein solution and crystallized repeatedly. The constituent proteins in the solution are determined spectrophotometrically. After 1 hour the enzymatic digestion is interrupted by raising the pH of the solution to 4.6 with 1N NaOH. The solution is then centrifuged for 30 minutes at 6000 rpm. After centrifugation all the HBsAg is contained in the supernatant, none being detectable in the pellet.

The digestion by pepsin causes the degradation of human plasma proteins into polypeptide units, whose size does not exceed 30,000 Daltons. The HBsAg protein is not digested.

The material from the pepsin digested plasma proteins is purified by the molecular filtering of particles up to 100,000 Daltons. A further closed-circuit filtering system may be employed, wherein the material to be filtered is repeatedly cycled through a molecular filtration system. Preferably the filter employed is a type H1 x 100 allowing the passage of particles up to 100,000 Daltons. The full filtering cycle is repeated nine times in a volume of 80 litres at a pressure of 6.2 atm.

Before the material is introduced into the filtering system, it is diluted to the volume specified above by the addition of phosphate buffered, deionized, apyrogenic water.

The filtering allows the complete removal of plasma proteins previously digested with pepsin. The material obtained from the filtering system, containing the purified HBsAg, is placed in a refrigerator in 10,000 ml portions for 96 hours, following the addition of formaldehyde such that its concentration in the solution is 1:2000 by volume. After removed from the refrigerator, the formaldehyde is removed from the material by means of dedialyzation. The HBsAg is then adsorbed on aluminium hydroxide which has been added in the ratio of 1 mg per 1 ml to the solution. The final vaccine obtained in this way is filled into ampoules.

The vaccine obtained in the way described above, when assayed in the presence of human plasma proteins, by double diffusion in agar, does not show precipitation lines at 30-fold thickening with plasma of anti-human proteins. Said vaccine also does not contain infectious particles of hepatitis B virus.

EXAMPLE

From a donor, HBsAg plasma is prepared by plasmaphoresis. The activity of HBsAg having a titre not lower than 1:16 is determined by means of immunoelectroosmophoresis. The human plasma is delipidized by adding 1 litre of 0.1 M $MnCl_2 \cdot 4\ H_2O$ and 30 Ml of solution containing 150,000 Int. units of heparin to 1 litre of plasma.

The said material is incubated at room temperature in an electromagnetic agitator for 30 minutes. The material is then centrifuged at 6000 rpm for 30 minutes. The pellet containing HBsAg/—/, is rejected and the supernatant is collected in a 2 litre flask.

To the material there is added 7.5% of polyethylene glycol solution, having a molecular mass of 6000. The pH is brought to 5.5 by the addition of 1N HCl. The material is then placed in a refrigerator and incubated over night under continuous stirring.

After 24 hours, the material is centrifuged at 6000 rpm. The supernatant, containing HBsAg/—/ is rejected and the pellet is dissolved in 200 ml of 10.9% NaCl and made up to 2 litres with deionized water. After 20—30 minutes a large amount of sediment appears which is removed by centrifugation at 6000 rpm for 30 minutes. Using immunoelectroosmophoresis the clear supernatant with the titre of 1:8 shows HBsAg activity. The volume of the material is then made up to 10 litres with deionized water. After 30 minutes of settlement a small amount of sediment forms which is removed by centrifugation at 6000 rpm for 30 minutes. Clarified supernatant is obtained, the titre whereof amounts has a ratio of 1:2 in the immunoelectroosmoprecipitation test.

To 10 litres of supernatant there is added 90 g of NaCl to produce a 0.9% NaCl solution.

The material prepared in this way is incubated at 37°C for 12 hours. At the end of 12 hours a pepsin solution at 37°C and pH — 2.5 is added in the ratio of 0.05 ml/ml, that is 0.5 g of pepsin per 10 litres of material. The pepsin digestion of the solution proceeds for 1 h at the temperature of 37°C. After 1 hour the digestion is stopped by increasing the pH to 3.5 by adding 0.1 N NaOH.

The remaining sediment is centrifuged at 6000 rpm for 20 min and discarded.

The supernatant undergoes molecular filtration with use of hollow fiber filters H 10 x 100.

In the molecular filtration 10 l of material is obtained, the titre whereof in the immunoelectroosmoprecipitation of HBsAg is of the ratio 1:2.

Said material at 100-fold thickening shows no precipitation lines in the immunodiffusion test on agar, using animal plasma:— anti-IgG, anti-IgM, anti-IgA, antihuman protein, and the plasma antihuman protein.

The vaccination of laboratory animals, such as guinea pigs or rabbits, with a 100-fold thickened material gives no immunological response against the contamination of the preparation with proteins originated from the human plasma. Only one precipitation line is observed with the HBsAg containing material.

The material obtained in the described method is highly immunogenic as proven by the vaccination of chimpanzees, and a dozen human volunteers.

Chemical characteristic of the vaccine for viral hepatitis B

HBsAG	— 0.5 um/cm³
Total protein	— 0.5 um/cm³
Polypeptides — molecular weight of 30,00	— 0.02 um/cm³
NaCl	— 0.85%
Aluminium hydroxide	— 1 mg/cm³
Merthiolate	— 100 um/cm³

In the material no HBcAG, HBeAG and DNA polymerase is detected when using the third generation tests.

CLAIMS

1. A method of preparing hepatitis B surface antigen comprising enzymatically degrading plasma proteins of human plasma to effect delipidization of the plasma and partial removal of plasma proteins digesting the degraded material with pepsin at a ratio of from 0.01 to 0.5 mg of pepsin per mg of protein, filtering the digested material on a molecular filter in a phosphate buffered medium of pH 7.1 to 7.3, recovering a

fraction of up to 100,000 Daltons and treating the said fraction with formaldehyde to disinfect same.

2. A method as defined in claim 1, characterized in that the initial material is digested by pepsin in the ratio of 0.05 mg/mg of protein.

3. A method as defined in claim 1, characterized in that the infectivity of the antigen purified from plasma proteins is inactivated with formaldehyde in the ratio of 1:2000 vol/vol for a time of 96 hours.

4. A method of preparing hepatitis B surface antigen according to the Example hereinbefore.

5. Hepatitis B surface antigen whenever prepared by the method claimed in any of claims 1 to 4.

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